Regulated proteolysis controls mucoid conversion in *Pseudomonas aeruginosa*

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Overproduction of the exopolysaccharide alginate causes mucoid conversion in *Pseudomonas aeruginosa* and is a poor prognosticator in cystic fibrosis. The ECF σ factor AlgU and its cognate anti-σ factor MucA are two principal regulators of alginate production. Here, we report the identification of three positive regulators of alginate biosynthesis: PA4033 (designated *mucE*), PA3649 (designated *mucP*), and *algW*. MucE, a small protein (9.5 kDa), was identified as part of a global *mariner* transposon screen for new regulators of alginate production. A transposon located in its promoter caused the overexpression of MucE and mucoid conversion in *P. aeruginosa* strains PAO1 and PA14. Accumulation of MucE in the envelope resulted in increased AlgU activity and reduced MucA levels. Three critical amino acid residues at the C terminus of MucE (WVF) were required for mucoid conversion via two predicted proteases AlgW (DegS) and MucP (RseP/YaeL). Moreover, as in *Escherichia coli*, the PDZ domain of AlgW was required for signal transduction. These results suggest that AlgU is regulated similarly to *E. coli* σ^E except that the amino acid triad signals from MucE and other envelope proteins that activate AlgW are slightly different from those activating DegS.

alginate | anti-σ factor | PDZ domain | protease cascade | signal specificity

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium, and is one of the most feared opportunistic pathogens infecting patients with compromised host defenses such as individuals with cystic fibrosis (CF). Chronic respiratory infections with *P. aeruginosa* are one of the major causes of morbidity and mortality in CF. During the course of lung infections in CF, *P. aeruginosa* colonizers often convert from low producers of the capsular polysaccharide alginate to high producers of alginate, resulting in the mucoid colony morphology. Isolation of mucoid *P. aeruginosa* strains is synonymous with clinical deterioration in CF and considered a poor prognosticator. The overproduction of alginate is thought to facilitate the formation of *in vivo* biofilms that helps to protect the bacteria from host defenses and antibiotic treatment.

A number of factors play a role in regulating alginate production in *P. aeruginosa* and their mechanisms of action have only partially been elucidated. Mutations in *mucA* of the *algU mucABCD* operon are a major cause of the conversion to the mucoid phenotype in clinical isolates from CF patients or laboratory isolates (1–3). MucA is an anti-σfactor located in the inner membrane that binds to and inhibits the alternative sigma factor AlgU (also called AlgT) from promoting the transcription of the alginate biosynthetic enzymes at the *algD* promoter (4–6). Induction of transcription of the alginate biosynthetic operons, *algD* and *algC* appears to be the key event leading to the production of alginate (7). Mutations in *mucA* have been found in a range from 44% to 84% of mucoid clinical isolates from CF patients (1, 2). The other members of this operon, MucB, MucC, and MucD, also appear to act in a negative manner along with MucA to inhibit AlgU activity. Expression of *algD* operon is regulated positively by some homologs of bacterial two-component systems called FimS-AlgR and KinB-AlgB and other transcription factors such as AlgZ (7, 8). Another alternative σ-factor RpoN, plays the dual role as both a positive and a negative regulator of expression of *algD* (9).

AlgU, MucA and MucB are homologous to proteins in the σ^E-mediated envelope stress response system in *Escherichia coli* (10, 11). σ^E is regulated principally by sequential proteolytic cleavage of the anti-σ factor RseA, first by DegS and then by YaeL/RseP (10). Stress such as elevated temperatures is thought to cause the misfolding of outer membrane (OM) proteins (OMPs) being assembled in the periplasm. DegS, which is activated by binding to the C-terminal YQF sequence present on many OMPs such as OmpC, cleaves off the C terminus of RseA. Removal of this C terminus renders RseA a substrate for RseP, which cleaves RseA within its transmembrane domain. The cleaved RseA is released from the membrane and rapidly degraded by cytoplasmic proteases, thereby eliminating it from inhibiting σ^E.

Because of the conservation between AlgU and σ^E, it seemed likely that AlgU was regulated by proteases and OMPs similarly to σ^E. But existing data were not consistent with this idea because AlgW (DegS) was reported to act negatively based on overexpression studies (12), and the only protease (Prc) implicated in degradation of MucA acted on truncated forms of MucA present in mucoid *mucA* mutants (13). In this article, we report the results of a global transposon mutagenesis screen we conducted in an attempt to identify additional factors that regulate AlgU activity and alginate production. Here, we will report how the investigation of a transposon mutation that occurred in the promoter region of an unknown gene has led to the identification of a small envelope protein, a potential signal for activating AlgU activity. Moreover, we show by deletion analysis that the two proteases orthologous to those degrading the *E. coli* anti-σ factor are required for signal transduction. Our results support a model in which AlgU is generally regulated in a similar manner as σ^E, with some differences between the two systems.

Results

Identification of a Positive Regulator of Alginate Synthesis. To identify regulatory genes for alginate synthesis, we used *mariner*-based transposition to conduct global mutagenesis on the nonmucoid *P. aeruginosa* reference strains PAO1 (14) and PA14 (15). After transposition, gentamycin-resistant mutants with a mucoid phenotype were isolated. The transposon insertion sites were mapped by inverse PCR. As expected, transposons insertions were identified within or near the coding regions of *mucA*, *mucB*, and *mucD*, previously known regulators of alginate synthesis. However, we identified two mucoid mutants with the same insertion from

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Abbreviations: CF, cystic fibrosis; OM, outer membrane; OMP, OM protein; PIA, *Pseudomonas* isolation agar.

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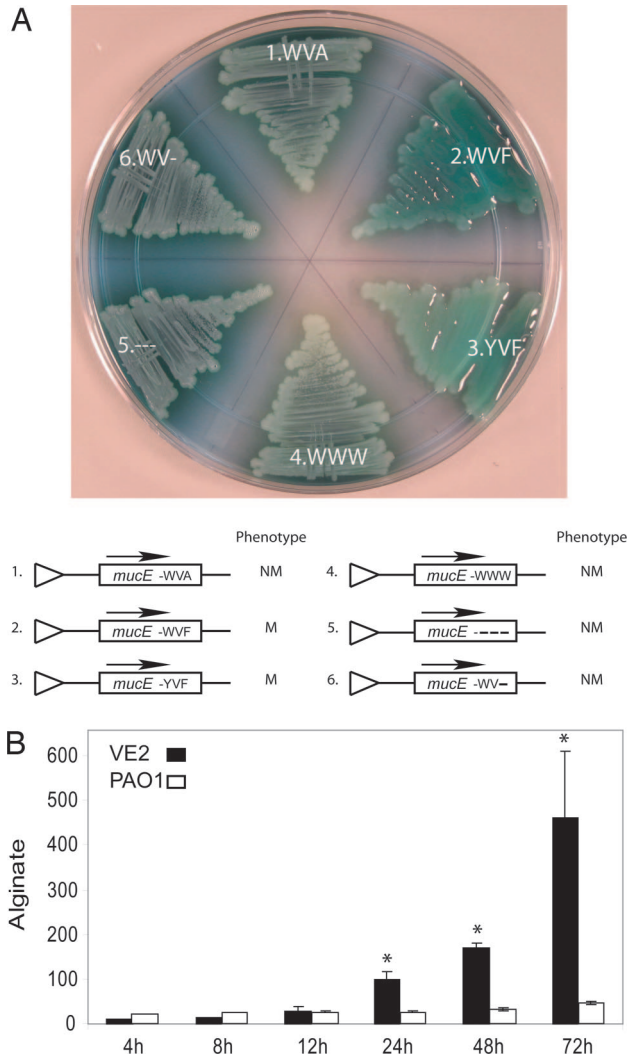

Fig. 1. The mucoid induction caused by the overexpression of MucE with different C-terminal motifs (*A*) and the effect of overproducing *mucE* on the alginate production in *P. aeruginosa* PAO1 (*B*). Plasmids contained *mucE* alleles with variation at the C-terminal residues fused to the *aacC1* (*Gm*^{r}) as found in PAO1VE2 and were introduced into the WT nonmucoid strain PAO1. The mucoid phenotype was scored after growth on PIA plates supplemented with carbenicillin for 24 h. NM, nonmucoid; M, mucoid. — denotes the deletion of the sequence corresponding to the last three amino acids at the C termini of MucE. WV−, deletion of the last amino acid (tryptophan–valine–no amino acid). WVF, tryptophan–valine–phenylalanine, the WT C-terminal sequence of MucE. VE2, the isogenic *mucE*-overproducing mutant of PAO1. For assaying alginate production, PAO1 and VE2 were grown on PIA plates at 37°C for indicated amount of time.

PAO1 (PAO1VE2) and PA14 (PA14DR4) that each had a *mariner* transposon inserted upstream of the coding region of a gene of unknown function (PAO1-PA4033 and PA14_11670) and which hereafter we called it *mucE*. These strains showed a large increase in alginate production as compared with the WT strains and had alginate levels similar to other mucoid strains (Fig. 1). The transposon insertions in PAO1VE2 and PA14DR4 were located upstream of the predicted Shine–Dalgarno sequence of *mucE* and placed the transposon encoded *aacC1* conferring Gm^r upstream of *mucE* [see supporting information (SI) Fig 5]. One explanation of our results is that the mucoid phenotype is due to the overexpression of MucE, and is thus a gain-of-function mutation. It has been reported that the *mariner* transposon can cause the overexpression of downstream genes because of its lack of a transcriptional terminator (16). These results suggest that MucE, if overexpressed, may act as a positive activator of alginate production.

Overexpression of MucE Causes Mucoid Conversion in PAO1 and PA14. We first tested whether transposon insertion resulted in overexpression of *mucE*. Using RT-PCR, we showed that the levels of *mucE* transcripts in the mutant strain PAO1VE2 were 7-fold higher than in the WT PAO1 strain, suggesting that the transposon insertion caused an increase in *mucE* transcription. Next, we sought to confirm that the *aacC1* of the transposon was responsible for the overexpression of MucE by cloning *aacC1* and *mucE* together as found in the chromosome of PAO1VE2 into the shuttle vector pUCP20 (17). We observed mucoid conversion when we introduced the resulting plasmid (pUCP20-*Gm*^{r}-*mucE*) into the nonmucoid strain PAO1, suggesting that the coexpression of *aacC1* and *mucE* caused mucoid conversion (Fig. 1*A*; 2-WVF). Next, we tested whether we could induce mucoid conversion by overexpression of *mucE* from heterologous promoters. Surprisingly, we did not see mucoid conversion of PAO1 when *mucE* was induced from a *lac* promoter (pUCP20-*mucE*) or a *tac* promoter (pVD*tac-mucE*) with the addition of 1–20 mM IPTG (SI Table 4). However, we did notice mucoid conversion when *mucE* was cloned behind a P_{BAD} promoter (pUCP20-P_{BAD}-*mucE*) and was induced by the addition of arabinose (SI Fig 6) and when *mucE* was expressed from the *aacC1* promoter without the *aacC1* coding region (pUCP20-P_{Gm}-*mucE* in SI Table 3). Similarly, we noted mucoid conversion when P_{Gm}-*mucE* was introduced into the chromosome of PAO1. The most likely explanation for these results is that mucoid conversion requires a high level expression of *mucE*, which can be achieved from the *aacC1* and P_{BAD} promoters. Finally, we tested whether introduction of pUCP20-*Gm*^{r}-*mucE* or pUCP20-P_{Gm}-*mucE* plasmids could cause mucoid conversion in other nonmucoid *P. aeruginosa* strains. We observed the emergence of a mucoid phenotype in the environmental isolate ERC-1 and nonmucoid clinical CF isolates including CF149 (18) and early colonizing strains (C0686C, C3715C, and C7406C, *mucA*$^+$) (SI Table 3). These results suggest that the overexpression of MucE can cause mucoid conversion and that MucE is an positive regulator for alginate synthesis.

The *mucE* Gene Encodes a Small Periplasmic or Outer Membrane Protein That Acts Through AlgW to Control the AlgU Activity. The *mucE* gene is predicted to encode a polypeptide of 89 aa with a probable transmembrane helix and a cleavable N-terminal signal sequence (14). Homologues of MucE are found in other species of pseudomonads capable of producing alginate (SI Fig. 7; Table 1). We confirmed that *mucE* encodes a protein by detecting an ≈10-kD protein in Western blots of cell extracts of *E. coli* and *P. aeruginosa* expressing His-tagged MucE (SI Fig. 8). PseudoCAP and Signal IP servers predicted that MucE is likely to be located in the periplasm. To test the localization of MucE, we constructed a series of deletions of *mucE-phoA* translational fusions. We observed phosphatase activity when *phoA* was fused to sequence corresponding to the full-length MucE or the N terminus after P36 but not after A25. The MucE C terminus–PhoA fusion did not show apparent phosphatase activity (SI Fig. 5). These results indicate that MucE is a small protein of 9.5 kDa located in the periplasm or outer membrane and the N-terminal signal sequence is required for translocation across the cytoplasmic membrane.

In *E. coli*, overexpression of OMPs that end with YXF motifs at the C terminus can induce the activity of σ^E, a homolog of AlgU (19–21). The C terminus of MucE is predicted to end with WVF and contains a conserved amphipathic β-sheet feature found in OMPs in other bacteria, suggesting that MucE may be an OMP that acts as a signal to activate AlgU activity (22). Therefore, MucE may operate through the AlgU-MucA pathway to regulate alginate production. To test this possibility, we first examined whether AlgU is required for MucE-mediated activation for mucoidy. We observed that the inactivation of *algU* in PAO1VE2 caused a loss of

Table 1. Effect of altering C-terminal signaling motif of MucE on mucoid induction in *P. aeruginosa* PAO1

Carboxyl terminal residues	Mucoid induction	OMPs with the same C-terminal residues
WVF (Wild-type)	M	MucE (PA4033)
YVF (−2 W to Y)	M	OprP (PA3279), OprO (PA3280)
LVF (−2 W to L)	M	MucE orthologue (*Pseudomonas fluorescens*)
WIF (−1 V to I)	M	MucE orthologue (*Pseudomonas syringae*)
WVW (0 F to W)	M	
WQF (−1 V to Q)	NM*	
YQF	NM*	OptS (PA2466), HasR (PA3408), OptQ (PA2355), and the major porins OmpC and OmpF of *E. coli*.
WLF (−1V to L)	NM	
DRF	NM	AlgE (PA3544)
YYF	NM	The strongest signaling motif in *E. coli*
YKF	NM	OprH (PA1178)
FQF	NM	AlgI (PA3548)
ELR (ΔWVF)	NM	
RWV (ΔF)	NM	
WVA (0 F to A)	NM	
WVY (0 F to Y)	NM	
WWW	NM	

M, mucoid; NM, nonmucoid.
*Slightly mucoid after 1 day of incubation.

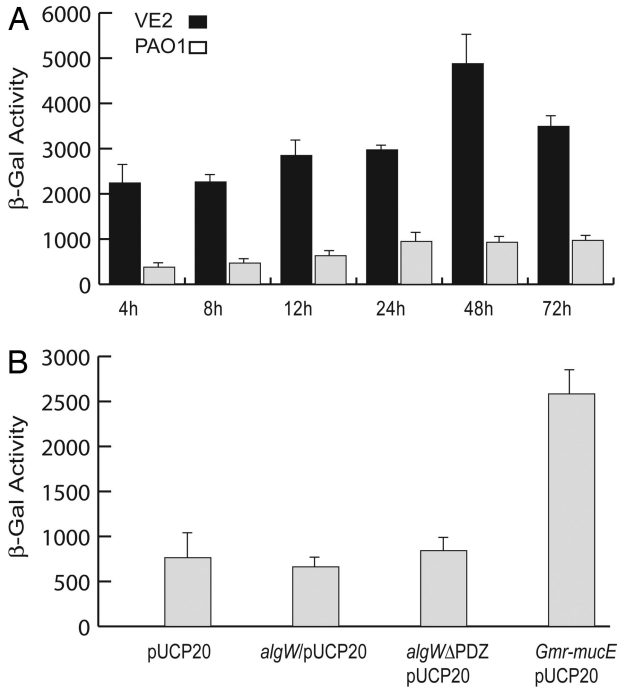

Fig. 2. The β-galactosidase activity (Miller Units) from the AlgU-dependent $P1_{algU}$-*lacZ* reporter on the chromosome in PAO1 and PAO1VE2 (*A*), and in PAO1 strains carrying different plasmids (*B*). The shown values represent the average from three independent experiments in which at least two measurements were conducted for each sample. The vector pUCP20 in *B* was used as control. Each of the strains was grown on PIA plates at 37°C for indicated amount of time (*A*) or 24 h before assaying (*B*).

mucoidy (Table 2). Next, we determined whether overexpression of MucE alters the activity of AlgU by measuring expression from the *algU* P1 promoter that is regulated by AlgU (23). We fused the *algU* P1 promoter to *lacZ* in the plasmid miniCTX and placed the gene fusion in the chromosome at the CTX phage attachment site (*attB*) in PAO1 and *mucE*-overexpressing strain PAO1VE2 (24). The β-galactosidase activity was significantly increased from 723.9 ± 259.6 units in PAO1 to 3116.2 ± 983.0 in the strain PAO1VE2 after 24 h of growth (Fig. 2). In addition, an increase in the cellular levels of AlgU was seen in PAO1VE2, as compared with PAO1 (Fig. 2), which is consistent with the autoregulatory role of AlgU. In addition, an increase in the cellular levels of AlgU was seen in PAO1VE2, as compared with PAO1 (Fig. 2 and SI Fig. 9). The in-frame deletion of *algU* blocked mucoidy of PAO1VE2, and the mucoid phenotype was restored to the PAO1VE2Δ*algU* strain carrying pUCP20-P_{BAD}-*algU* by addition of 0.1–1% of arabinose. These results indicate that AlgU activity is required and is increased when MucE is overexpressed.

C-Terminal WVF Motif Is Critical for MucE-Induced Mucoidy. MucE contains a C-terminal sequence of WVF, which is similar to the C-terminal YXF motif of OMPs that activates DegS and σ^E activity. We tested whether these C-terminal residues of MucE affected its ability to induce mucoidy. We constructed pUCP20-Gm^r-*mucE* derivatives in which either one or three C-terminal resides of MucE had been altered or deleted (Table 1). We observed that a construct lacking the last residue (ΔF) of MucE or the last three residues of MucE (ΔWVF) did not cause mucoid conversion of PAO1 (Fig. 1). These results suggest that unfolded C terminus of MucE may activate a protease to cleave MucA in a similar manner as OMPs activate DegS to cleave RseA. In *E. coli*, it was observed that activation of DegS to cleave RseA is induced by OMPs ending in YQF or YYF. Therefore, we tested whether MucE ending with these residues could function similarly by increasing the activity of AlgU causing mucoid conversion by we constructed variant MucEs ending in YQF or YYF. Only slight mucoid conversion was noted when MucE ended in YQF and no mucoid conversion was seen for MucE ending with YYF (Table 1). These results suggest that the signal that activates AlgU activity in *P. aeruginosa* may be different from the one that activates σ^E. Lastly, we sought to determine what residues were allowed on the C terminus of MucE to induce mucoid conversion. We constructed a series of plasmids that overexpress MucE with different C-termini and observed their effect on mucoid appearance (Table 1). It is concluded that F and W residues were allowed at the C-terminal position because YVF and WVW caused conversion to mucoidy. Similarly, V or I were allowed at the −1 position and W, Y, or L at the −2 position.

AlgW Is Essential for MucE-Mediated Mucoid Induction. The observation that overexpression of MucE with C-terminal residues WVF inducing AlgU activity and mucoidy led us to search for a protease similar to DegS in *E. coli*. Presumably, this protease would be activated to cleave MucA upon its PDZ domain binding to the C-terminal WVF sequence of MucE. It is clear that DegS is activated to cleave RseA by binding to C-terminal of OMPs ending with YXF (20). A BLAST homologue search indicated that the protein within the *P. aeruginosa* genome showing the highest similarity to DegS is encoded by *algW* (PA4446) (SI Fig. 10). The orthologous relationship of DegS and AlgW had also been noted (10), but a previous study suggested that AlgW did not act in a similar manner as DegS (12). Boucher *et al.* (12) reported that *in trans* overexpression of *algW* suppressed mucoidy in three *P. aeruginosa* strains with *mucA* mutations, indicating that AlgW acted to repress alginate production. To test whether AlgW is a positive regulator of AlgU, acting similarly to DegS, we disrupted the *algW* gene in the PAO1VE2 strain that overexpressed *mucE*. We generated two mutants: *algW*::tet^R and the other with in-frame deletion of *algW* (PAO1VE2Δ*algW2*). Both were nonmucoid. Inactivation

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Table 2. Phenotype of the key mutants produced in this study and the effect of *mucE*, *algW*, *algU*, and *mucP* on the mucoid conversion in *P. aeruginosa* PAO1 and PA14

Strains	Genotype	Phenotype	Plasmid and its effect on the NM-to-M conversion	
			Plasmid	Phenotype
PAO1	Prototroph	NM	pUCP20 (vector)	NM
PA14	Prototroph	NM	pUCP20	NM
PAO1VE2	PAO1 with chromosomal fusion of P_{Gm}-*aacC1-mucE*	M	pUCP20	M
PA14DR4	PA14 with chromosomal fusion of P_{Gm}-*aacC1-mucE*	M	pUCP20	M
PAO1VE2Δ*algU*	PAO1VE2 with in-frame deletion of *algU*	NM	pUCP20-P_{BAD}-*algU*	M*
			pUCP20	NM
PAO1VE2Δ*algW*	PAO1VE2 with in-frame deletion of *algW*	NM	pUCP20-*algW*	M
			pUCP20	NM
PAO1VE2Δ*mucP*	PAO1VE2 *mucP*::*tet*^{R}	NM	pUCP20-*mucP*	M
			pUCP20	NM

M, mucoid; NM, nonmucoid.
*The PAO1VE2Δ*algU* strain harboring pUCP20-P_{BAD}-*algU* remained NM without the addition of arabinose.

or deletion of *algW* was responsible for the loss of mucoidy because the mucoid phenotype was restored in PAO1VE2Δ*algW* strain by *in trans* complementation with pUCP20-*algW*. Similarly, the disruption of *algW* in PAO1 (PAO1Δ*algW*) prevents the mucoid induction even when plasmid-borne *mucE* (pUCP20-*Gm*^{r}-*mucE*) was in a state of overexpression. These results indicate that the *algW* gene is required for the MucE induced mucoidy and that AlgW is a positive regulator of alginate. Our results also suggest that the C terminus of MucE may activate AlgW to cleave MucA, which is similar to the C-terminal residues of *E. coli* OMP's activating DegS to cleave RseA.

PDZ Domain of AlgW Acts as a Sensor and Inhibitor of Proteolysis. The PDZ domain of DegS, acts as both a sensor of misfolded proteins and inhibitor for its protease activity (20). This was suggested by the observation that the deletion of the PDZ of DegS caused cleavage of the anti-σ factor RseA and slight activation of σ^E activity in the absence of activating signals, suggesting that the PDZ acts to inhibit the protease activity in the absence of misfolded OMPs signal. Because of the similarity between AlgW and DegS, we decided to test whether the PDZ domain of AlgW works in a similar manner. Therefore, a truncated *algW* gene was constructed in pUCP20 that lacked the 3′ sequences encoding the PDZ domain of AlgW (pUCP20-*algW*Δ*PDZ*). When pUCP20-*algW*Δ*PDZ* was introduced into PAO1, we did not observe mucoid conversion. Because the loss of the PDZ domain in DegS only caused a slight activation of σ^E activity, we next examined the effect of AlgWΔPDZ expression on AlgU activity by measuring transcription from the AlgU-dependent P1 promoter. We observed a slight but significant increase ($P < 0.05$) in β-galactosidase activity from the *algU*-P1 promoter-*lacZ* fusion in cells expressing AlgW lacking the PDZ domain compared with the cells with the vector or expressing full-length *algW* (pUCP20-*algW*Δ*PDZ* vs. pUCP20 or pUCP20-*algW* in Fig. 2). On the other hand, the pUCP20-*algW*Δ*PDZ* could not restore the mucoid phenotype to the PAO1VE2Δ*algW* strain like pUCP20-*algW* did. These results suggest that the PDZ domain of AlgW may act as both a sensor and inhibitor of proteases activity like the PDZ domain of DegS.

AlgW Is Involved in the Cleavage of Anti-σ Factor MucA upon MucE Overexpression. If AlgW acts in the same manner as DegS, it should be able to cleave the anti-σ factor MucA, leading to its degradation and preventing it from inhibiting AlgU. We first tested whether overexpression of MucA from the *lac* promoter in pUCP20-*mucA* could suppress the mucoid conversion caused by the overexpression of MucE. When pUCP20-*mucA* was introduced into PAO1VE2, the mucoid phenotype was completely suppressed as opposed to the control vector with no effect. Second, we examined the levels of MucA in PAO1, MucE-overexpressing strain PAO1VE2, the isogenic *algW* mutant (PAO1VE2Δ*algW*). A reduced level of MucA protein in PAO1VE2 was noted as compared with PAO1 and PAO1VE2Δ*algW* (Fig. 3). In contrast, the levels of AlgU, which is cotranscribed with *mucA,* were higher in PAO1VE2 than in PAO1 (Fig. 3). Critically, in PAO1VE2, which lacks AlgW, no changes were seen in the levels of MucA or AlgU under these conditions (data not shown). These results support a notion that AlgW acts as a protease that cleaves MucA upon activation. The activation of AlgW can be achieved by the misfolded MucE because of overexpression or stress, leading to a reduction in MucA levels and an increase in AlgU levels.

MucP (PA3649) Is Also Essential for MucE-Induced Conversion to Mucoidy. In *E. coli*, the degradation of RseA requires another protease called RseP (also known as YaeL) to cleave the anti-σ factor RseA after it is cleaved by DegS (25, 26). The *P. aeruginosa* genome also contains a homolog of RseP (PA3649, designated as MucP) (SI Fig. 11). We wanted to determine whether MucP played a role in the degradation of MucA and activation of AlgU activity. We first tested whether the loss of MucP would affect alginate production. We observed that inactivation of *mucP* in PAO1VE2 caused a loss of mucoidy (PAO1VE2Δ*mucP* in Table 2). Furthermore, the plasmid pUCP20 (pUCP20-*mucP*) could restore the mucoid phenotype in PAO1VE2Δ*mucP*. Similarly, disruption of *mucP* in PAO1 prevented mucoid conversion when a high level of MucE was present from plasmid pUC20-*Gm*^{r}-*mucE*. In addition, we detected a higher level of MucA and a lower level of AlgU in PAO1VE2Δ*mucP* as compared with PAO1VE2 (data not shown).

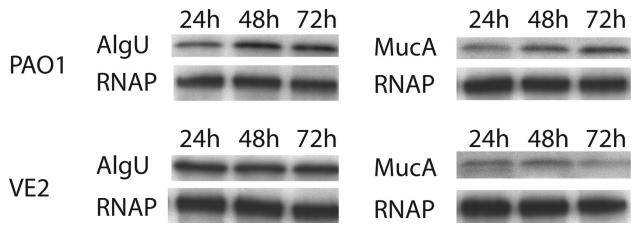

Fig. 3. Western blots displaying MucA levels were reduced in the MucE-overexpressing strain PAO1VE2 as compared with the WT PAO1. The levels of AlgU and MucA protein were collected from PAO1 and PAO1VE2 cells as grown on PIA plates for the indicated amount of time. RNAP, antibodies against RNA polymerase α-subunit. The sources of antibodies are as indicated in *Materials and Methods*.

These results indicate that MucP is required for MucE activation of AlgU activity.

MucE-Induced Mucoidy Does Not Require the Prc Protease. The gene *prc* (PA3257) was recently identified as a regulator of alginate synthesis in *P. aeruginosa* and is predicted to encode a PDZ domain-containing periplasmic protease similar to a *E. coli* protease called Prc or Tsp (13). Prc appears to act to promote mucoidy in *mucA* mutants by degrading truncated forms of MucA found in mucoid *mucA* mutants (13). To test whether Prc plays a role in the activation of alginate production mediated by MucE, we tested whether overexpression of MucE can induce mucoidy in a strain lacking Prc. We observed that cells of the *prc* null mutant PAO1–184 (*prc*::*tet*^{R}) carrying either MucE overexpression plasmid pUCP20-*Gm*^{r}-*mucE* or pUCP20-P$_{Gm}$-*mucE* were as mucoid as PAO1 cells carrying pUCP20-*Gm*^{r}-*mucE* or pUCP20-P$_{Gm}$-*mucE*. These results suggest that Prc is not required for mucoidy induced by MucE and is consistent with Prc only acting against truncated forms of MucA.

MucD Can Eliminate Signal Proteins That Activate AlgW and Other Proteases to Cleave MucA. The *mucD* gene (PA0766) is a member of the *algU mucABCD* operon and is predicted to encode a serine protease similar to HtrA in *E. coli* (12). MucD appears to be a negative regulator of mucoidy and AlgU activity (12). Our *mariner* transposon library screen confirmed this result because several mucoid mutants were isolated that had transposons inserted within the coding region of *mucD* (PAO1VE19 in SI Table 3). HtrA in *E. coli* has been hypothesized to regulate the σ^E stress response system by removing misfolded proteins in the periplasm that can activate the DegS protease via the degradation of the anti-σ factor RseA (25, 26). Therefore, we examined whether MucD of *P. aeruginosa* acted in a similar manner as HtrA of *E. coli*. To test this, we first examined whether overexpression of MucD would suppress the mucoid phenotype in a strain overexpressing MucE. Overexpression of *mucD* from the plasmid pUCP20-*mucD* partially suppressed the mucoid phenotype of the *mucE*-overexpressing strain PAO1VE2. This result is consistent with the notion that MucD can aid in the elimination of misfolded OMPs including MucE. Next we tested whether the mucoidy caused by the loss of MucD required AlgW and MucP. As expected, we observed that disruption of *mucP* in the mucoid *mucD* mutant PAO1VE19 caused the loss of the mucoid phenotype (PAO1VE19Δ*mucP*, SI Table 3). The mucoid phenotype of PAO1VE19Δ*mucP* was restored when *mucP* was *in trans*. However we did not see a loss of the mucoid phenotype from the *mucD* mutant PAO1VE19 after the disruption of *algW* (PAO1VE19Δ*algW*, SI Table 3). Our results suggest that MucD can act to remove misfolded proteins that activate proteases for degradation of MucA and that at least under certain conditions other proteases independent of AlgW could also initiate the cleavage of MucA.

Discussion

Through a transposon mutant screen, we identified MucE (PA4033) as a positive activator of alginate production and AlgU activity. Our results indicate that MucE acts as an envelope signal that activates AlgW and perhaps other proteases to cleave MucA in a manner similar to the way OMPs in *E. coli* regulate σ^E (19). We confirmed that *mucE* encodes a protein that is secreted into the periplasm because of an N-terminal signal peptide. MucE may localize to the outer membrane after the translocation because its C termini are similar to the conserved amphipathic β-sheet commonly found on OMPs in other bacteria (22). In *E. coli*, it has been shown that the accumulation of misfolded OMPs can lead to the activation of σ^E activity (19, 20). The C termini of OmpC and OmpF can bind to the PDZ domains of the periplasmic protease DegS to activate DegS for cleavage of the C terminus of RseA, the anti-σ factor of σ^E. This promotes elimination of RseA and activation σ^E (19, 20). Our identification of MucE as an activating signal also allowed us to determine that the predicted proteases AlgW (PA4446) and MucP (PA3649) play a positive role in regulating AlgU activity and alginate production. AlgW and MucP are likely to act in a manner similar to *E. coli* DegS and RseP, respectively. Presumably AlgW is activated by binding to the C termini of MucE to cleave off the C terminus of MucA thereby promoting the sequential cleavage of MucA by MucP, leading to the degradation of MucA and the release of active AlgU (Fig. 4).

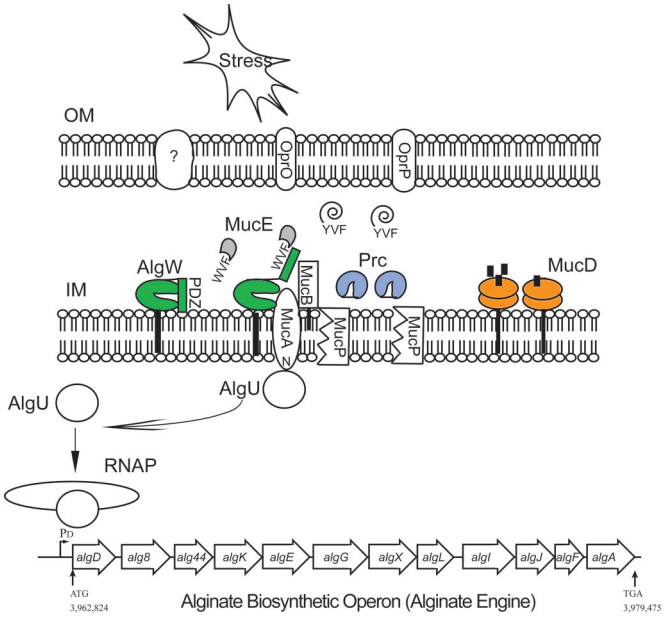

Fig. 4. Schematic diagram for signaling pathway involved in regulation of alginate synthesis and mucoid conversion. External stresses affect the folding of periplasmic proteins (PPs) and OMPs bearing the C-terminal signaling motifs such as WVF and YVF and may also increase their expression. The exposed signaling motifs bind the PDZ domain and exert an allosteric activation on the protease domain of AlgW, initiating the cleavage of anti-σ factor MucA, followed by the cleavage by the second protease MucP. The released σ-factor AlgU undergoes autoregulation and induces the overproduction of alginate, conferring a mucoid phenotype displayed by many clinical isolates from CF patients.

The C-terminal residues that activate AlgU activity appear to be different from the residues that activated σ^E activity in *E. coli*. In *E. coli*, the cleavage of RseA by DegS is activated by OMPs ending in -YXF motifs (20). We observed that overexpression of MucE ending with WVF, YVF, LVF, WIF, or WVW resulted in the mucoid phenotype, but MucE ending with YYF or YQF, which are strong activators of σ^E activity in *E. coli* (20), caused no mucoid conversion or a weak conversion, respectively. Therefore the protease(s) that presumably act to cleave MucA appear to be activated by different C-termini than the C termini recognized by DegS in *E. coli*. This observation probably reflects differences in the OMPs found in *P. aeruginosa* as compared with *E. coli*. *P. aeruginosa* is noted for its low OM permeability to various antimicrobial compounds, because of the relative low frequency of general-purpose porins and lack of the homologues of OmpC and OmpF, two major OMPs of *E. coli* (27). To determine whether other proteins besides MucE might activate AlgU activity, we examined the C termini of known and probable OMPs in the *P. aeruginosa* genome (SI Table 5). *P. aeruginosa* cells generally express between five and nine major OMPs, depending on the conditions of growth (28). The most abundant OM porin in *P. aeruginosa* cells is OprF. OprF (-EAK) lacks the

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conserved C-terminal motif found in most other OMPs. Another major OM porin that is expressed in both mucoid and nonmucoid strains is OprH (29, 30). OprH ends with YKF, a sequence that did not induce mucoidy when placed on the end of MucE (Table 2). However, OprP and OprO end with the sequence YVF which was observed to induce mucoidy when fused to *mucE* (Table 2). In addition, two other probable OMPs (OprB and OprB2) in *P. aeruginosa* end with the sequence TVF, which was not tested as a potential inducer for mucoidy, but has a good possibility for full induction of mucoidy like WVF. Most of the other known or probable OMs proteins of *P. aeruginosa* belong to the OprD family or TonB family. Although none of these predicted proteins contain C-termini that have been shown to induce mucoidy they do contain sequences that have conservative substitutions from WVF and so may be able to induce mucoidy (SI Table 5). Therefore it seems likely that a few other OMPs beside MucE would be able to activate AlgU activity presumably by binding to the PDZ domain of AlgW. However, *P. aeruginosa* may not use some of its most abundant OMPs such as OprF and OprH as the envelope signals. Because alginate is rapidly produced by the WT *mucA*$^+$ strains of *P. aeruginosa* at the early stage of lung infection (31), MucE may represent one of the major signaling molecules induced before the selection of the *mucA* mutants in the CF lungs.

In conclusion, we have identified MucE, MucP and AlgW as positive regulators of AlgU which is regulated in a similar manner as σ^E of *E. coli*. Both of these ECF sigma factors appear to be regulated by misfolded OMPs activating protease(s) to cleave their cognate anti-σ factor. The regulated proteolysis machinery is preserved to control the nonmucoid to mucoid conversion in *Pseudomonas*. However the protein motif that activates the proteases in each system appears to be different. Furthermore, the proteolytic degradation of the anti-σ factor may be a common mechanism used to regulate ECF σ-factors in bacteria.

Materials and Methods

Bacterial Strains, Plasmids, and Transposons and Growth Conditions. Bacterial strains, plasmids, and transposons used in this study are shown in SI Table 3. *P. aeruginosa* strains were grown at 37°C in Lennox broth (LB), on LB agar or *Pseudomonas* isolation agar plates (PIA; Difco, Sparks, MD). Whenever necessary, the PIA plates were supplemented with carbenicillin, tetracycline, or gentamicin at a concentration of 300 μg/ml.

Transposon Mutagenesis. The mariner transposon-containing plasmid pFAC (32) was introduced into PAO1 and PA14 by biparental conjugations. The locations of the transposon insertion of the mucoid mutants were determined by inverse PCR. The chromosomal DNA of these strains was digested with SalI and ligated to generate circular closed DNA molecules (Fast-Link DNA ligation kit; Epicentre, Madison, WI). The ligated DNA was then used as the template for inverse PCR using primers that anneal to the gentamycin resistance gene (Gm^r), and the amplicons were sequenced.

Mutant Construction and Complementation Analyses. The *Pseudomonas* mutants were created by inserting an antibiotics resistance cassette into the specific gene or by in-frame deletion as described (17, 24, 33). These genes were cloned into the shuttle vector pUCP20 for complementation assay and the gene expression driven by *lac*-promoter.

Biochemical Assays. Alginate levels, β-galactosidase, and alkaline phosphatase activity were measured by using the established methods as described in *SI Text* (34, 35).

Western Blot Analyses. Cell lysates were prepared by using Ready-Preps kit (Epicentre) and electrophoresed on SDS/PAGE gels. The blots were probed by using published or commercially available mouse monoclonal antibodies against AlgU (3), His-tag (Qiagen, Valencia, CA), or rabbit polyclonal antibodies against MucA (36) or RNA polymerase α subunit (13). HRP-labeled goat anti-mouse IgG or HRP-labeled anti-rabbit IgG was used as the secondary antibodies. Enhanced chemiluminescence ECL (Amersham Biosciences, Piscataway, NJ) was used for detecting HRP-labeled goat anti-mouse IgG or anti-rabbit IgG (Roche, Indianapolis, IN).

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